BioNanoSci.
DOI 10.1007/s12668-016-0272-3

CRISPR/Cas9 Technique for Identification of Genes Regulating Oxaliplatin Resistance of Pancreatic Cancer Cell Line

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Abstract Genome editing approach based on prokaryotic CRISPR (clustered regularly interspaced short palindromic repeats) system is a simple and useful way to investigate gene functions on a genome-wide scale. It is especially important for cancer research because of genetic contribution to tumor development. We applied this technique in a high-throughput screening format to find genes that could be involved in chemotherapy resistance of pancreatic cancer. We used AsPC1 cell line expressing doxycycline-inducible Cas9 to screen two sgRNA lentiviral libraries: (1) cell cycle genes (CC, 983 genes, ~12,000 sgRNA) and (2) genome-wide (GW, ~90,000 sgRNA). These sets of cells with different gene knockouts were treated with oxaliplatin to identify knockouts which increase sensitivity to the drug. We have performed screening both in vitro and in vivo settings. For the in vivo arm of our experiments, peritoneal carcinomatosis model in severe combined immunodeficiency (SCID) mice was created by intraperitoneal injection of AsPC1/Cas9 cells infected with sgRNA library. Genomic DNA from cells and animal tumor material was analyzed using next generation sequencing (NGS) to obtain data about representation of sgRNA. Preliminary data allowed us to identify genes potentially modulating oxaliplatin sensitivity.

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Published online: 08 October 2016

Keywords Genome editing · CRISPR/Cas9 · Pancreatic cancer · Drug resistance · Oxaliplatin

1 Introduction

Tumor drug resistance is one of the main problems in clinical oncology, especially in pancreatic cancer therapy [1]. Only 5–20 % of pancreatic cancer cases respond to the first-line therapy with gemcitabine [2, 3]. Pancreatic cancer also has the worst survival rate of all cancers [1]. According to the American Cancer Society, the 5-year relative survival rate for all stages of pancreatic cancer is 6 %. Finding genes involved in drug resistance mechanisms is necessary to increase treatment efficiency. This information can also be used for development of personalized pancreatic cancer diagnostics and therapy.

A new genome edition technology CRISPR/Cas9 [4, 5] has become a powerful tool for more detailed study of gene functions in norm and pathology, including cancer diseases. This technology is based on a prokaryotic immune system providing protection against exogenous genetic material by making double-strand breaks (DSBs) in a phage DNA. The main components of the CRISPR/Cas9 system are an endonuclease Cas9 and a molecule of a single-guide RNA (sgRNA) that has 20-base pairs (bp) region complementary to a target genome sequence and directs Cas9 to a gene of interest. DSBs in genomic DNA of mammalian cells are repaired by different mechanisms, including non-homologues end-joining (NHEJ) that results in reading frame shifts and indel (insertions or deletions) mutations. Mutations can cause loss of function (or knockout) if DSB occurs in a coding exon [6]. CRISPR/Cas9 technology can be applied in both in vitro and in vivo settings.

High-throughput CRISPR/Cas9 screening allows to analyze a large set of gene knockouts under the same experimental conditions [7]. sgRNA libraries (a mix of oligonucleotides for a set of genes) are designed and used to create lentiviral libraries, where each lentiviral particle contains one sgRNA. These lentiviral libraries are used for the infection of cell line expressing the doxycycline-inducible Cas9. A lentiviral cassette with one sgRNA will be integrated into genomic DNA of each cell. After doxycycline induction of Cas9 expression, DSBs are created depending on the gene specific sgRNA in each cell. As a result, there is a set of cells with knockouts of different genes that can lead to the alteration of the cell phenotype, or death if a mutated gene is essential for cell viability. Subsequent data analysis of sgRNA representation obtained from NGS allows to correlate phenotype changes with the knockouts [6, 7].

To date, several sgRNA libraries have been created for genes involved in different cell processes, such as cell cycle, kinases, nuclear proteins, and ribosomal genes [7]. CRISPR/Cas9 screening technique has been used for the identification of genes involved in mouse non-small-cell lung cancer metastasis development [6], genes essential for cell viability in cancer [7, 8] and pluripotent stem cells [8], and also those involved in resistance to vemurafenib in a melanoma model [8].

We used CRISPR/Cas9 loss-of-function screening to identify genes that could be modulating oxaliplatin sensitivity in pancreatic cancer cell line (Fig. 1a). The main idea is that, under treatment conditions, knockout of genes responsible for drug resistance results in death of cells carrying these knockouts and loss of the corresponding sgRNA. Subsequent comparison of sgRNA content from oxaliplatin treated and untreated samples allowed identification of candidate genes regulating sensitivity to the drug.

2 Material and Methods

sgRNA Libraries Two sgRNA libraries were used: (1) cell cycle proteins genes (CC, ~12,000 sgRNA) taken from Addgene, and (2) genome-wide (GW, ~90,000 sgRNA) was synthesized by CustomArray Inc.. Both libraries included 100 negative control sgRNA and ~1900 positive sgRNA. The sgRNA sequences were taken from Wang [7].

Cell Culture Pancreatic cancer cell line AsPC1 expressing doxycycline-inducible Cas9 was grown in DMEM/F12 media supplemented with 10 % of fetal bovine serum, L-glutamine, non-essential amino acids, penicillin and streptomycin. Minimal handled amount of cells was 5×10^6 to provide 400× and 55× coverage of CC and GW libraries respectively.

In Vitro CRISPR/Cas9 Screening AsPC1/Cas9 cells infected by appropriate sgRNA library were cultured with 1 μg/ml doxycycline to induce Cas9 expression and then treated with oxaliplatin (1 μM final concentration) during nine cell divisions (12 days). After that, genomic DNA was extracted from cells, and sgRNA-containing regions were amplified and barcoded by PCR for further distribution analysis by NGS according to Wang [7].

In Vivo CRISPR/Cas9 Screening in Xenograft Model Settings 3×10^6 AsPC1/Cas9 cells infected by CC library

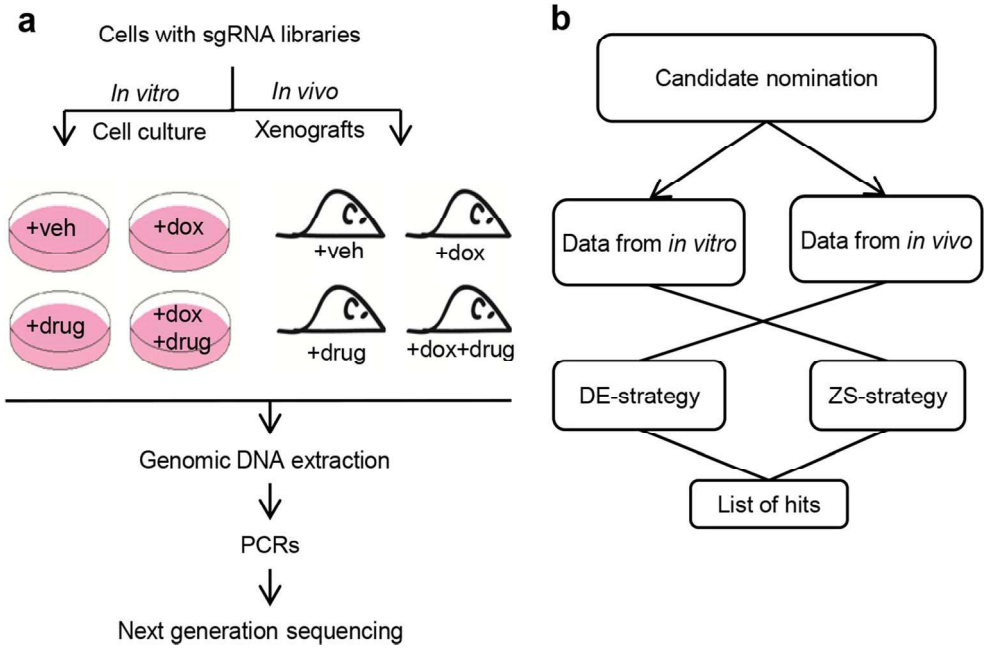

Fig. 1 Design of experiment. **a** General scheme of in vitro and in vivo screenings. **b** Scheme of data analysis

were intraperitoneally injected into SCID mice to create a peritoneal carcinomatosis model. Experimental animal group got a doxycycline-containing food and 4 mg/kg oxaliplatin intraperitoneally once a week during 3 weeks. At the end of the experiment, animals were sacrificed by CO_2 asphyxiation. Ascites and intestine serosa with tumors were used for genomic DNA extraction and subsequent sgRNA containing regions amplification as described.

3 Results and Discussion

Data analysis from primary screens allowed us to identify genes potentially regulating oxaliplatin sensitivity. Two statistical strategies were used to analyze each in vitro and in vivo screening data (Fig. 1b) as previously described [9]. The first DE-strategy is based on negative binomial generalized linear model, and the second ZS-strategy is based on *Z*-score. Genes were binned based on number of techniques nominating them, and mean sensitizing index (SI) was calculated for each bin (Fig. 2a). We observed a trend (Fig. 2a, black line) for genes with more robust experimental support to have a higher mean SI, indicating that we can use the robustness in reproducibility for the stratification of our hits for the subsequent validation. This conclusion was also supported by our analysis of hits enrichments with genes known to be involved in Pt-based drug resistance, which were extracted from the Comparative toxicogenomics database (CTD, http://ctdbase.org/), GeneCards database (http://www.genecards.org/), and from Bartz [10]. For each information source, our experimental candidates supported by two or more methods have a higher enrichment with known platinum drug sensitivity genes (Fig. 2b). Gene ontology analysis of hits was also performed (Fig. 2c), with the results consistent with the data reported in the CTD and GeneCards databases.

We consider our results as proof of feasibility, and our data need to be further validated. On the next stage of work, we are planning to validate gene hits using CRISPR/Cas9 loss-of-function screen with focused sgRNA library, RNA, and CRISPR interference approaches.

4 Conclusions

As a result of this study, we have nominated 130 candidate genes, which could be involved in the modulation of oxaliplatin resistance. Further investigation will help to identify among them new markers that can be used for diagnostics and treatment optimization of pancreatic cancer and also can be used as targets for new chemotherapy development.

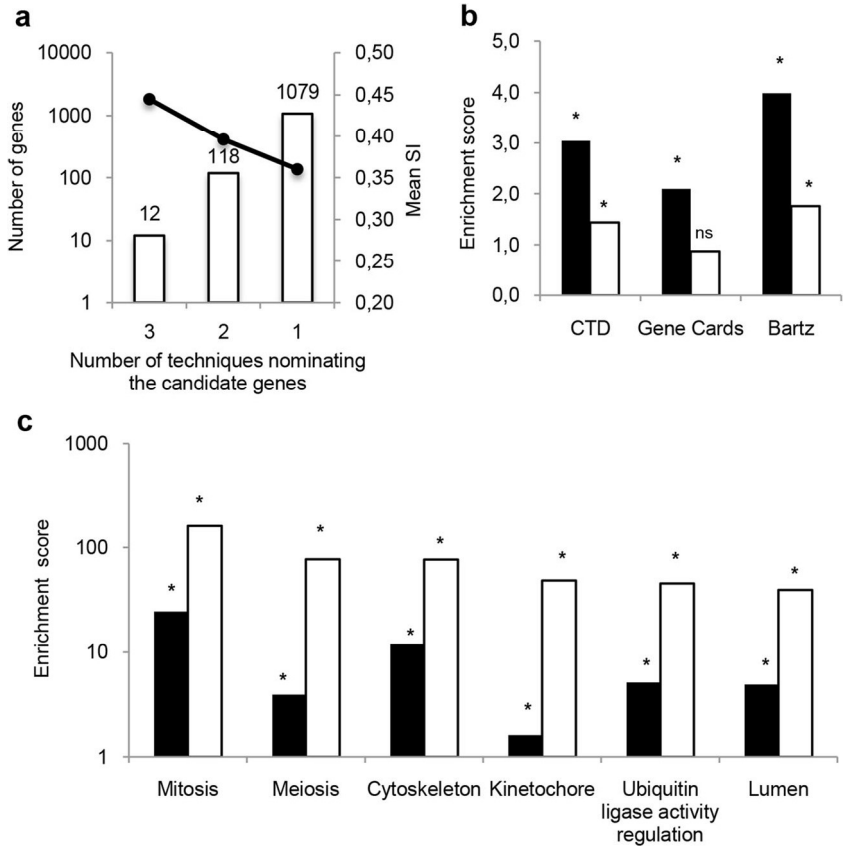

Fig. 2 Analysis of data. **a** Primary characterization of candidate hits. *Bars* show a number of genes, supported by one, two, or three statistical methods. The *black curve* shows a mean sensitivity index (SI) for each group. **b** Hits enrichment with genes involved in Pt-based drug resistance according to indicated databases and Bartz [10]. *Black bars* reflect enrichment for genes supported by two or more statistical methods, *white bars* reflect enrichment for genes supported by one statistical method. $*p < 0.05$; *ns* non significant. **c** Gene ontology analysis. *Black bars* reflect functional enrichment for genes supported by two or more statistical methods, *white bars* reflect functional enrichment for genes supported by one statistical method. $*p < 0.05$

Acknowledgments This study was supported by the Russian Science Foundation (project 15–15–20032).

Compliance with Ethical Standards All experiments were performed according to protocols approved by the FCCC institutional animal use committee and Ethic committee of Kazan Federal University.

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